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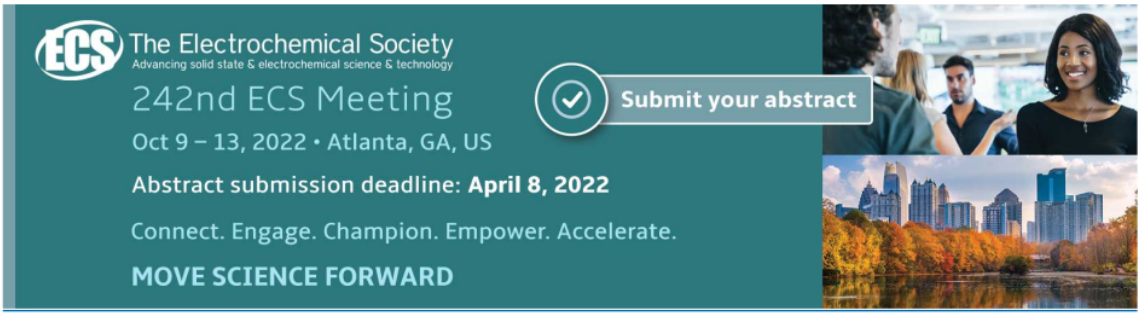

Characterization of hydroxyapatite by cytotoxicity test and bending test

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Abstract. Hydroxyapatite has been synthesized as a bone graft material by analyzing the membrane made according to the cytotoxicity test and bending test. The method used is dry ball milling. Cytotoxicity testing was carried out by making isolated osteoblast cells cultured in a 75 cm^2 cell flask containing a minimum of essential media. Based on the cytotoxicity test, it was found that hydroxyapatite is not toxic with cell viability above 60% (non-toxic requirements ISO 10993-5). From the variation of the hydroxyapatite mixture used, the average cell viability was (100.48 ± 11.98)%. A mixture of 2.5% Ag and 5% Ag with hydroxyapatite also provides good viability, which is above 60%, which means it is not toxic. The results of the analysis of the bending test and the compressive test showed that the larger the hydroxyapatite mixture, the lower the bending and compressive test results. The maximum bending test on a 10% mixture is 22.1 M.Pa and the compressive test on a 5% mixture is 16 ^{o}ShD.

1. Introduction

Hydroxyapatite (HAp) is one of the bioactive materials for bone. The biomaterial is a natural or man-made material that is used to interact directly with biological systems. The use of this biomaterial aims to repair, replace damaged tissue. Biomaterials based on the type of material can be metals, polymers, ceramics, and composites [1]. The application of biomaterials is for medical use such as bone and tooth grafts or guided tissue regeneration membranes [2].

At this time the need for biomaterials is very high and has had a considerable impact, especially in the field of orthopedic medicine, for example for bone repair, both in the repair of fractured and broken bones. The materials used in the treatment must be bioactive, biocompatible, and non-toxic [3].

Hydroxyapatite ($Ca_{10}(PO4)_6(OH)_2$) is synthesized from calcium sources derived from calcium oxide (CaO). Calcium oxide can be obtained from the calcination of $CaCO_3$ contained in natural materials such as limestone. CaO powder is dissolved with water to be converted into calcium hydroxide $Ca(OH)_2$. $Ca(OH)_2$ solution is mixed with a phosphoric acid solution to obtain a source of phosphate as the main ion forming hydroxyapatite [4].

Hydroxyapatite has bioactive properties against body tissues, can function in conjunction with body tissues, and can occur perfect osteointegration, no local and systemic toxicity, and no genotoxic activity in the body's biological system [5]. Hydroxyapatite has been widely used to repair, fill, add and reconstruct damaged bone and tooth tissue and in soft tissue [6]. Hydroxyapatite can be produced from bone and limestone.

Graft bone is a bone replacement material that can be used as a healing material for bone damage after going through certain stages. Bone grafts can be used to repair broken bone (fractures) and as a joint to prevent bone shifting. The synthetic bone graft material is a biomaterial that has bioactive properties (resulting in the formation of a direct chemical bond to bone), osteointegration (causing a strong bond between bone and implant), osteoconductive (becoming a place for new bone regeneration), biocompatible (fitting bone), bioresorbable (the surface that can be overgrown with tissue), has pores so that a better bond with the tissue can be obtained and is not toxic. Osteoconductive and osteointegration of bone grafts are related to the level of porosity and pore size [7]. Bone grafts that are widely used are the types of autograft, allograft, xenograft, and synthetic graft [8]. The material that best meets the requirements to become a synthetic bone graft is hydroxyapatite [9].

Hydroxyapatite crystals have the same size as bone hydroxyapatite crystals, which range from 20-50 nm. Hydroxyapatite has a hexagonal crystal structure with cell dimensions a = b = 9.42 Å and c = 6.88 Å. Stochiometrically Ca/P hydroxyapatite has a ratio of 1.67 and is chemically the same as a human bone mineral. Hydroxyapatite is the main inorganic component of bone tissue. Due to the similarity of chemical structure with human bone tissue minerals, synthetic hydroxyapatite shows good affinity, that is, it can chemically bind to bone [9], [10].

Bending strength or flexural strength is the greatest bending stress obtained from external loading without experiencing large deformation or failure. The magnitude of the bending strength depends on the type of material and loading. The bending test results in the upper part of the specimen experiencing stress, while the lower part will experience tensile stress. In composite materials, the compressive strength is higher than the tensile strength. Since the specimen is unable to withstand the tensile stress it receives, it will fracture, failing the composite test. The bending strength at the top is the same as the bending strength at the bottom. The test carried out is three-point bending [11].

In research Annur, they used hydroxyapatite material in bending testing because it has the same chemical composition as hard tissues in humans such as teeth and bones. This study was to determine the value of the bending stress with variations in volume fraction of hydroxyapatite 40% HAp, 50% HAp, 60% HAp, and 70% HAp. The bending test is carried out using the ASTM D790 standard using the three-point bending test method. The results obtained are the maximum bending stress value of 31.2 MPa on the specimen with 50% hydroxyapatite percentage volume fraction [12].

In research Sirait has synthesized limestone to obtain hydroxyapatite by the ball mill method and precipitation [13]. The wet method is a commonly used method of producing HAp because it is simple and can produce HAp powder which is mostly amorphous. In the precipitation method, the synthesis is carried out using a liquid reaction. Phosphorus solution is dripped little by little into the calcium solution to produce HAp with high purity and very small particle size. The properties and characteristics of HAp depend on the method and source of the constituent elements. The results obtained are hydroxyapatite with a size of 66.05 nm, a hexagonal structure, and a Ca/P ratio of 1.25. The smaller the hydroxyapatite particle size, the wider the contact area between the implant and the surrounding tissue. so that the obtained bond can be better. This hydroxyapatite product was used as a bone graft composite material. The membrane was made using the MTT (Mikrotetrazolium) method with variations of hydroxyapatite and Ag, then characterized by cytotoxic test, bending test, and compressive test.

2. Research Methods

Cytotoxicity testing was carried out by making isolated osteoblast cells (MC3T3-E1 cell line ATCC CRL-2594, Virginia, USA) cultured in a 75 cm^2 cell flask containing the minimum essential medium (MEM, Gibco, Massachusetts, USA) then added with 10% fetal bovine serum (FBS, Gibco, Massachusetts, USA) and 1% antibiotic-antimycotic (Corning, New York, USA) and placed in an incubator with a humidity of 5% CO_2 atmosphere at 37 °C. To determine the effect of cell viability, 500 µL of MC3T3-E1 cell suspension was taken and put into a 24-well plate with a cell density of 2 x 10^4 cells/plate and incubated for 1 day to allow the cells to adhere. Furthermore, the toxicity of MC3T3-E1 cells was given hydroxyapatite (HAp), 2.5% Ag-HAp, 5% Ag-HAp with various concentrations

(25µg/mL, 50µg/mL, 100µg/mL, 150µg/mL and 200µg/mL) in the extracted solution. Samples on well plates without extraction were used as controls. Each sample concentration was prepared in triplicate.

After 3 days of incubation, samples were taken from all well-plates and 200 µL of thiazolyl blue tetrazolium bromide MTT solution (L119139, Alfa Aesar, Massachusetts, USA) was added to each well-plate and incubated for another 4 hours to form formazan. At the end of the incubation period, the MTT solution was replaced with 300 µL of dimethyl sulfoxide (DMSO, ECHO, Taiwan) added to each well-plate. After that, the solution was transferred to a 96-well plate and its absorbance was measured at 570 nm using a microplate reader (Multiskan Go, Thermo Scientific, USA). The percentage of cell viability of each sample was calculated assuming control cell viability as 100%.

A flexibility test was carried out by making samples using the dry ball milling method and the matrix used was Polymethyl methacrylate (PMMA). PMMA powder is mixed with hydroxyapatite (HAp) powder with various compositions (PMMA: HAp) is (100:0)%; (95:5)%; (90:10)%; (80:20)% and in ball milling for 12 hours. After mixing well, 6 mL of PMMA (liquid) was added and stirred evenly to obtain a gel. After the gel is formed, then the gel is poured into a mold with a size of (60 x 10 x 6) mm according to the ASTM D790 standard and allowed to dry. For the hardness test, it was formed with a size of (30 x 10 x 6) mm (ASTM D2240) and tested for hardness using a Shore D Durometer.

3. Results and Discussion

3.1. Cytotoxicity Test

HAp is a bioceramic in the medical field is similar to the mineral phase in bone and teeth, so it has bioactive biocompatibility properties, which allows surrounding tissue to grow into the implant and the presence of porosity so that a better bond with the tissue can be obtained. In addition, there are several additional potential advantages, including low electrical and thermal conductivity, elastic properties similar to bone, and being able to function as a barrier layer when using metallic materials as bone implant substrates. The application of HAp in the medical field is usually as a coating for metal-based implant materials to increase their biocompatibility [14].

Biocompatibility is defined as the ability of a material to provide a good biological response when applied to the body [15]. Cytotoxicity testing of a material is an early stage in determining the biocompatibility of implant materials. The MTT test is one of the methods used in the cytotoxicity test and is a quantitative test to determine the level of cytotoxicity in vitro and indicates the presence of nontoxicity in porous hydroxyapatite samples. Determination of the number of cells that survive the cytotoxicity test can be done in various ways. There is a determination based on the parameters of membrane damage, impaired synthesis and degradation of macromolecules, modification of metabolism, and changes in cell morphology.

The results of cytotoxicity testing using MTT on hydroxyapatite samples with various concentrations of 25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml, and 200 µg/ml, and doped with a silver (Ag) obtained Figure 1. Figure 1 shows that the more HAp mixture, the higher the cell viability, meaning that with the increase in HAp, the tissue is less toxic. At a mixture of 150 and 200 µg/ml HAp obtained constant viability, this needs to be studied again for the increase in the above mixing, whether it decreases or not. When viewed from the mixing of 2.5% Ag-HAp, the cell viability decreased, but this was inversely proportional to the mixing of HAp but the optimal result was at a concentration of 25 µg/ml. In the 5% Ag-HAp mixture, the higher the concentration made, the lower the viability, but still above the permissible standard with an average of 94.87%.

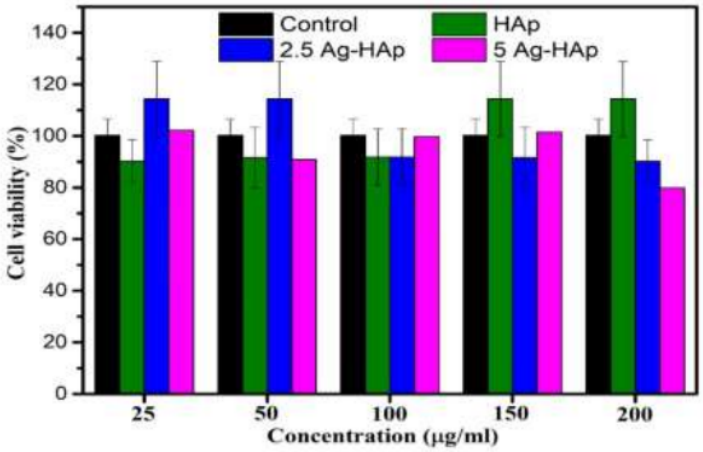

Figure 1. Histogram of cell viability with the MTT method

The percentage of hydroxyapatite living cells with a concentration of HAp 200 µg/ml, 150 µg/ml was above the control value of 114.38%, while the concentrations of 100 µg/ml, 50 µg/ml, and 25 µg/ml were below the control value of 91,21%. But all samples were above the grade limit of 70%, which means the sample had a good percentage of live cells. The percentage of living cells produced from the MTT test proved that HAp compounds were not toxic to fibroblast cells [16].

According to the standard [17] a material is not toxic if its viability is above 60%. From the graph above, the percentage of live cells resulting from the MTT test is above 70% and this includes grade 3 (ISO 10993-5). The graph of the MTT test results showed that HAp was biocompatible and non-toxic as a bone graft material because the growth of living cells exceeded the toxic limit of 70%. Not toxic to fibroblast cells (cell line) because the percentage of cell viability is still above 60%, namely Optical Density (OD) which states the high and low growth of bacterial cells in the given medium [18].

3.2. Bending Test Results

The results of the bending test can be seen in Figure 2. From the results of the bending test, it was found that the addition of HAp powder reduced the bending strength from 39.1 to 19.9 M.Pa for the addition of 5% HAp powder. The highest bending strength for the mixture of HAp powder with PMMA matrix was 22.1 MPa, namely in the sample with a composition of 10% HAp powder and 90% PMMA matrix.

These results indicate that the addition of HAp will cause a reduction in the bending strength of the matrix used, however, it needs to be studied again because this decrease fluctuates. It is necessary to pay attention to the mixing of the ingredients so that they are completely homogeneous and the mixing tim 15 Synthetic hydroxyapatite has a very low mechanical strength compared to bone [19]. One way to improve the mechanical properties of HAp is to use metal as a coating, polymer composite with ceramics [20], [21]. The bending test is the largest bending stress that can be accepted due to external loading without experiencing large deformation or failure. The magnitude of the bending strength depends on the type of material and loading.

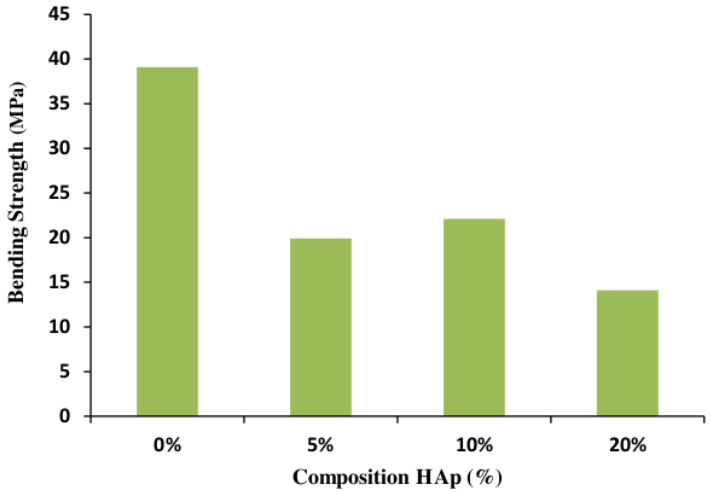

Figure 2. The Histogram of Bending Test

From the graph above, it can be seen that the specimens with HAp percentages of 0%, 5%, 10%, and 20% have fluctuating bending stresses, this is due to the placement of the specimens which is done manually and does not use a chuck so that when given a load it has the possibility the specimen may shift and the load may fall at an undesirable point. Besides being caused by the placement of the specimen, the fluctuating stress value can be caused by the manufacture of specimens where the hydroxyapatite powder used is not evenly distributed on the test specimen, resulting in the bending stress value of the hydroxyapatite test having a fluctuating value [12].

3.3. Hardness Test Results

The compressive strength or hardness test is the maximum resistance of the sample to a given pressure until damage occurs to the sample because it is no longer able to provide a given load. The value of the compressive strength of the sample is influenced by the applied stress. The compressive strength test was carried out on standard specimens of the test rods. The material to be tested is first made into a test rod with a shape according to certain standards. In the middle of the test rod is the part that receives the stress, in this part the length of the test rod is measured, that is the part that is considered to receive the effect of loading.

Compression test using Durometer Shore D and the data are presented in Figure 3. From the figure, it can be seen that the addition of hydroxyapatite to the matrix makes the hardness decrease.

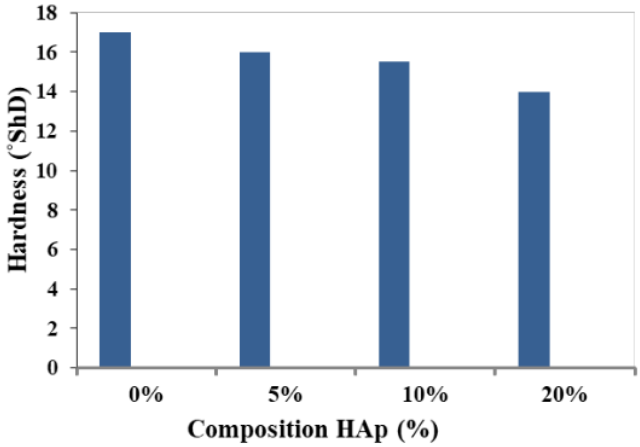

Figure 3. Histogram of Hardness Test Results

The higher the degree of ShD indicates a higher level of hardness. Based on the results of the hardness test, it was found that with the addition of HAp powder, the hardness level decreased from 17 °ShD to 14 °ShD with a percentage of 20% HAp.

4. Conclusion

Based on the cytotoxicity test, it was found that hydroxyapatite is not toxic with cell viability above 60% (non-toxic requirements ISO 10993-5). From the variation of the hydroxyapatite mixture used, the average cell viability was (100.48 ± 11.98)%. The higher the concentration, the higher the viability, the maximum viability in a mixture of 200 µg/ml. A mixture of 2.5% Ag and 5% with hydroxyapatite also provides good viability, which is above 60% which means it is not toxic, the average cell viability is (100.48 ± 11.98)%. The average is the same as without the Ag mixture, but the higher the concentration, the lower the viability, the maximum viability in the mixture of 25 µg/ml. The results of the analysis of the bending test and the compressive test showed that the larger the hydroxyapatite mixture, the lower the bending and compressive test results. The maximum bending test on a 10% mixture is 22.1 M.Pa and the compressive test on a 5% mixture is 16 °ShD**.**

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